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Factors Affecting the Multiplication and Subculture of *Treponema pallidum* subsp. *pallidum* in a Tissue Culture System

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Limited multiplication of *Treponema pallidum* subsp. *pallidum* (Nichols strain) can be obtained in the presence of Sf1Ep rabbit epithelial cell cultures, but continuous culture has not yet been achieved. In the system currently employed, growth is exponential for the first 10 to 15 days of culturing, after which multiplication and the percentage of motile organisms decrease. In an effort to identify culture conditions which may adversely affect treponemal viability and growth, eight culture parameters were monitored over a 12-day period of incubation. Several of these parameters, including pH, redox potential, dissolved oxygen concentration, and glucose levels were found to change dramatically during the course of incubation, indicating that they may be responsible for the cessation of treponemal multiplication. The feasibility of extending the period of growth by subculturing was also investigated. In preparation for planned serial subcultivation experiments, several subculture procedures were tested and found to be effective in allowing the transfer of *T. pallidum* from 3-day-old primary cultures to secondary cultures without loss of motility or growth potential. Increases of up to 55-fold were observed in secondary cultures, but increased growth due to subculturing was not a consistent finding. Use of subculture intervals of ≥6 days resulted in a progressive decrease in treponemal multiplication in secondary cultures, although retention of motility was extended in the subcultures compared with motility in the primary cultures. These results indicate that the lack of continued multiplication of *T. pallidum* in subcultures is not due to damage to the treponemes during subculture. Prolonged multiplication of *T. pallidum* may be obtained through the stabilization of culture conditions by either performing subcultures at regular intervals or by medium replacement techniques. It was also found that primary *T. pallidum* cultures could be established by using as the inoculum treponemes that had been stored at −70°C in a medium containing 15% glycerol.

Treponema pallidum, the causative agent of syphilis, is one of the few bacteria pathogenic to humans which has not yet been cultivated continuously in vitro, necessitating the use of organisms cultured in rabbits for research and diagnostic purposes. In 1981, a major barrier was overcome when consistent multiplication was obtained in a tissue culture system developed by Fieldsteel et al. (2, 3). In contrast to previous reports of *T. pallidum* cultivation, the results obtained by this group were highly reproducible and have been corroborated by other laboratories (5, 6). In this system, *T. pallidum* extracted from infected rabbit testes is added to Sf1Ep cottontail rabbit epithelial cell cultures containing a modified tissue culture medium with 20% fetal bovine serum (FBS) and 0.63 mM dithiothreitol (DTT). The cultures are incubated in a microaerobic atmosphere at 33 to 34°C. The treponemes attach to the Sf1Ep cells and multiply with a doubling time of 30 to 50 h; up to 100-fold increases in the number of organisms have been obtained. Growth in primary cultures is limited to the first 10 to 15 days of culture and typically reaches a maximum of 1×10^8 to 5×10^8 treponemes per 10 ml (36 cm^2) of culture. The Fieldsteel culture system has already been useful in determining the MICs of antibiotics against *T. pallidum* (S. J. Norris and D. G. Edmondson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, A68, p. 12; Norris and Edmondson, manuscript in preparation), and the suitability of in vitro-cultured *T. pallidum* for serologic tests for syphilis is currently under investigation.

The multiplication of *T. pallidum* after transfer to secondary cultures has not been reported previously. Development of a procedure in which *T. pallidum* could be grown continuously (or for prolonged periods) would greatly enhance the applicability of the Fieldsteel system to studies on the physiology, immunology, and pathogenicity of *T. pallidum*. Therefore, continuous culture of *T. pallidum* by either subculture or by continuous-flow techniques is the principal goal of our current studies.

The complex set of conditions required for *T. pallidum* multiplication is responsible for the difficulty encountered in obtaining consistent in vitro multiplication, which was first reported 76 years after the organism was first identified. The restrictive growth conditions, sensitivity to the toxic effects of oxygen, slow multiplication rate, and required presence of metabolically active tissue culture cells combine to limit treponemal multiplication and culture life in primary cultures (1, 4, 7). These same factors would be expected to have an even more deleterious effect on attempts to maintain the bacterium continuously in vitro.

In this study, an attempt was made to identify factors responsible for the cessation of treponemal growth in primary cultures and to examine the feasibility of subcultivation as a means of prolonging survival and multiplication. This information will be useful in determining the best approaches to continuous culture.

MATERIALS AND METHODS

Reagents. Tissue culture medium components were purchased either from Flow Laboratories, Inc., McLean, Va., or GIBCO Laboratories, Grand Island, N.Y. Trypsin was obtained from Sigma Chemical Co., St. Louis, Mo., and DTT was obtained from Calbiochem-Behring, LaJolla, Calif. Calf serum (Hyclone Laboratories, Inc., Logan, Utah) was

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used for routine maintenance of cell cultures. A specific lot of FBS (Hyclone lot 100315; Hyclone Laboratories) was used in all *T. pallidum* cultivation experiments. All sera were heat inactivated at 56°C for 30 min before use.

Tissue culture. Sf1Ep rabbit epithelial cells were originally obtained from either A. H. Fieldsteel or from the American Type Culture Collection, Rockville, Md., and were between passages 74 and 89 at time of use. They were maintained as previously described (S. J. Norris and D. G. Edmondson, Sex. Transm. Dis., in press) and were trypsinized and seeded at 2.5×10^5 or 5×10^5 cells in 8oz (1oz = 29.573 ml) prescription bottles 2 days before *T. pallidum* cultivation studies.

In vitro cultivation of *T. pallidum*. Primary cultures of *T. pallidum* were established as described previously (2; Norris and Edmondson, in press) and were incubated at 33 to 34°C in a microaerobic chamber containing 1.5 to 2.0% O_2, 5% CO_2, and 93 to 93.5% N_2 (Norris and Edmondson, in press). The medium in the Sf1Ep cultures was replaced with 10 ml of pregassed basal reduced minimal medium (BRMM) with 20% FBS and was equilibrated in the microaerobic chamber for 3 to 5 h before inoculation with *T. pallidum*. The inoculum consisted of approximately 5×10^6 to 1×10^7 *T. pallidum* in 0.34 ml of the extraction medium. The concentration was determined by dark-field microscopy, using a Helber counting chamber (Hawksley and Sons Ltd., Lancing, West Sussex, England [Norris and Edmondson, in press]).

Monitoring of culture parameters. In most experiments, duplicate or triplicate cultures were harvested by trypsinization, and the concentration and motility of *T. pallidum* were quantitated by dark-field microscopy (Norris and Edmondson, in press). In the experiments in which changes in culture parameters were examined, the following protocol was used, again using duplicate or triplicate cultures for each time point. Redox potential and dissolved oxygen concentration were measured within the microaerobic chamber to avoid the effects of atmospheric oxygen levels on these determinations. Redox potential was measured directly in the culture flask with a combination platinum electrode. A 5-ml sample of medium then was transferred by pipette from the culture to a beaker to measure dissolved oxygen concentration with an oxygen electrode (model 97-08; Orion Research, Inc., Cambridge Mass.). A Clark-type electrode with a Teflon membrane separating the medium from the electrode was used to prevent electrochemical interactions (e.g., from reducing agents) from affecting the readings. Care was taken during these measurements to minimize mixing and thus avoid introduction of gaseous oxygen into the medium.

Cultures were removed from the chamber, and pHs were measured immediately with a Ross combination electrode (model 810300; Orion Research, Inc.). A sample of medium was saved for glucose and sulfhydryl compound concentration determinations. Cultures then were harvested, and *T. pallidum* was quantitated as described above. The number of Sf1Ep cells per culture was determined by bright-field microscopy using a hemacytometer.

The concentration of reduced sulfhydryl groups was assayed by the reduction of benzofuroxan (8). A 0.1-ml volume of the culture medium was added to a solution containing 1.0 mM benzofuroxan, 1.0 mM EDTA, and 6.7% ethanol in a $NaHCO_3$-NaOH buffer with a pH of 9.6 and an ionic strength of 0.1. The assay mixture was incubated at 25°C for 12 min, and A_{430} was measured with a Gilford spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio). Controls consisted of BRMM medium with 20% FBS to which various concentrations of a freshly prepared DTT solution were added. A linear relationship existed between DTT concentration and A_{430}. Glucose concentrations were determined by using the Centrifichem glucose-hexokinase clinical assay (Baker Instruments Corp., Allentown, Pa.) in conjunction with a Multistat III microcentrifugal analyzer (Instrumentation Laboratory Inc., Lexington, Mass.).

Subculture procedures. Transfer of *T. pallidum* from primary to secondary cultures was performed within the microaerobic chamber by using treponemes from two to four primary cultures for each subculture. Secondary cultures were prepared in the same manner as primary cultures, except that 0.34 ml of infected testes extract (passed through a 0.22-μm-pore filter to remove residual *T. pallidum* and used either fresh or stored at −20°C) was added to each culture. All media were preequilibrated with 1.5% O_2–5% CO_2–93.5% N_2 before use. For the supernatant method, the medium from the primary cultures was simply removed and pooled. The *T. pallidum* concentration was determined, and the volume containing the desired inoculum was added to the secondary cultures. In the mechanical disruption method, all but 3 ml of the culture medium was removed, and the cell layer was detached from the flask with a rubber policemen. The flask was rinsed with 2 ml of fresh BRMM with 20% FBS, which was added to the culture suspension. The suspension was drawn through a pipette several times to further dissociate attached treponemes from the Sf1Ep cells, and then the suspension was centrifuged at approximately $250 \times g$ for 2 min to sediment the tissue culture cells. The supernatant was counted and used for inoculation. The procedures for the trypsin plus EDTA, EDTA alone, and EDTA plus fetal bovine serum protein fraction (P2) methods were identical except for the solution used for culture dissociation. In each case, the basal medium was Ca^{2+}- and Mg^{2+}-free Eagle minimal essential medium with nonessential amino acids and 0.63 mM DTT. Trypsin and EDTA were each added to a concentration of 0.2 mg/ml of culture, and P2 was used at a concentration equivalent to 20% serum (~6 mg of protein per ml). The medium was removed from the cultures, and the cell monolayer was rinsed briefly with 2 ml of the dissociating medium. A 3 ml volume of the dissociating medium was added to each flask, and then all flasks were incubated at 33 to 34°C for 20 min without shaking. Each of the media tested resulted in efficient release of the Sf1Ep cells from the glass and in release of treponemes from the surface of the rabbit cells. The dissociated cultures were pipetted vigorously and removed, and the flask was rinsed with 2 ml of fresh BRMM with 20% FBS. The culture suspension and final BRMM rinse were pooled and centrifuged at $250 \times g$ for 2 min. The concentration of *T. pallidum* in the supernatant was quantitated, and the appropriate volume was added to the secondary cultures. All secondary cultures were incubated in the microaerobic chamber under the same conditions used for primary cultures.

RESULTS

Changes in culture conditions. The parameters shown in Table 1 were assessed on days 0, 3, 6, 9, and 12 in cultures of *T. pallidum* incubated with Sf1Ep rabbit epithelial cells. In these experiments, treponemal growth and motility were maintained at high levels during the first 9 days of incubation. The doubling times were 67 h for days 1 to 3, 45 and 42 h for days 3 to 6 and 6 to 9, respectively, and 288 h for days 9 to 12. Thus, the growth pattern appeared to exhibit the lag, exponential, and stationary phases typical of most bacterial

TABLE 1. Changes in culture conditions during in vitro cultivation of *T. pallidum*

Day of incubation	Parameter, mean ± SEM[a]							
	T. pallidum per culture (10^6)	Motility (%)	Sf1Ep cells/ culture (10^5)	pH	Redox potential (E_{cal})	Reduced sulfhydryl (μM)	% Dissolved O_2 (pO_2)	Glucose (mg/ml)
0	5.2 ± 1.1	99.7 ± 0.4	1.5 ± 0.4	7.60 ± 0.05	−26 ± 18	69 ± 8	1.52 ± 0.06	2.06 ± 0.12
3	11.0 ± 0.3	90.3 ± 3.3	3.0 ± 2.0	7.49 ± 0.07	−7 ± 15	36 ± 5	1.27 ± 0.16	1.87 ± 0.09
6	33.1 ± 9.3	96.0 ± 2.1	7.3 ± 2.3	7.38 ± 0.13	4 ± 12	39 ± 1	1.11 ± 0.19	1.46 ± 0.16
9	111 ± 25	97.0 ± 0.7	8.8 ± 3.7	7.14 ± 0.08	11 ± 8	41 ± 5	1.16 ± 0.16	0.81 ± 0.08
12	132 ± 42	73.0 ± 20	8.3 ± 2.1	6.93 ± 0.06	6 ± 11	43 ± 3	0.72 ± 0.16	0.33 ± 0.10

[a] Three separate experiments. pO_2, Partial oxygen pressure.

cultures. The decrease in motility from 97% on day 9 to 73% on day 12 coincided with the decrease in the rate of multiplication. The number of Sf1Ep cells increased from 1.5×10^5 per culture to $>8 \times 10^5$ per culture, corresponding to a change in percent confluency of approximately 20 to 100%.

Several physical parameters were found to change during the course of incubation (Table 1). Day 0 values were obtained at the time of inoculation and therefore represent control values which can be used for comparison with values for the later time points. The pH decreased from 7.6 to below 7.0, whereas the redox potential rose from its initial highly reduced state. The concentration of reduced sulfhydryl groups, measured by benzofuroxan assay, varied little during incubation. However, most of the DTT, which was initially present in the medium at a concentration of 0.63 mM, apparently was oxidized during the preequilibration period prior to inoculation with *T. pallidum*. The concentration of dissolved oxygen present in the medium decreased to half its inital level during the 12-day period of incubation. Glucose levels exhibited the most dramatic change, dropping from an initial concentration of 2.06 to 0.33 mg/ml on day 12.

Comparison of subculture procedures. A variety of subcultivation techniques were used to determine whether *T. pallidum* could be successfully transferred from primary to secondary cultures. The simplest of these involved transferring the culture medium, which contained between 15 and 50% (mean ± standard error of the mean = 27 ± 3%) of the total *T. pallidum* per culture. However, most of the treponemes were attached to the surface of the Sf1Ep cells; therefore, methods capable of dissociating *T. pallidum* from the mammalian cells were also tested. These included mechanical disruption (with a rubber policeman) and treatment with trypsin plus EDTA, EDTA alone, or EDTA plus P2 (Norris and Edmondson, in press). Each of these subculture procedures resulted in the efficient release of *T. pallidum* from the Sf1Ep cells, thus providing a uniform suspension which could be quantitated and transferred to secondary cultures in an accurate and reproducible manner. The EDTA plus P2 procedure was included because *T. pallidum* does not survive well in the absence of serum. P2 is capable of replacing the serum requirement of *T. pallidum* without introducing Ca^{2+} and Mg^{2+}, which would interfere with the detachment of *T. pallidum* because of EDTA chelation of divalent cations (Norris and Edmondson, in press).

In the initial experiments, day 3 or 5 primary cultures were used as the source of *T. pallidum* for subculture. These short subculture intervals were chosen to minimize the effects of other variables affecting treponemal viability (such as depletion of nutrients or accumulation of toxic products), so that any decrease in the growth rate in secondary cultures would most likely be due to adverse conditions introduced by the subcultivation procedures.

The results of a representative experiment are shown in Fig. 1. Secondary cultures were inoculated with 1.3×10^6 to 4.2×10^6 *T. pallidum* from day 3 primary cultures. The growth rates for the secondary cultures were quite similar to those of the primary cultures, indicating that none of the procedures tested adversely affected the multiplication of *T. pallidum*. Maximal yields in the subcultures ranged from 4.9×10^7 to 9.9×10^7, reflecting a 21- to 55-fold increase in the number of *T. pallidum* in the secondary cultures.

A summary of the results obtained in five subculture experiments is given in Table 2. Overall, the total fold increase in the number of *T. pallidum* was 25 ± 4 (standard error of the mean; range, 19 to 39) in the primary cultures and 37 ± 6 (range, 9 to 99) in the secondary cultures, including the multiplication occurring in the primary cultures before transfer. Within individual subcultivations, the degree of multiplication was as much as 2.5 times greater than that in the primary cultures. However, this trend was not consistent from experiment to experiment, and the difference between the subculture and primary culture yields was not statistically significant ($P > 0.1$). Treponemal multiplication appeared to be comparable for all of the subculture methods tested.

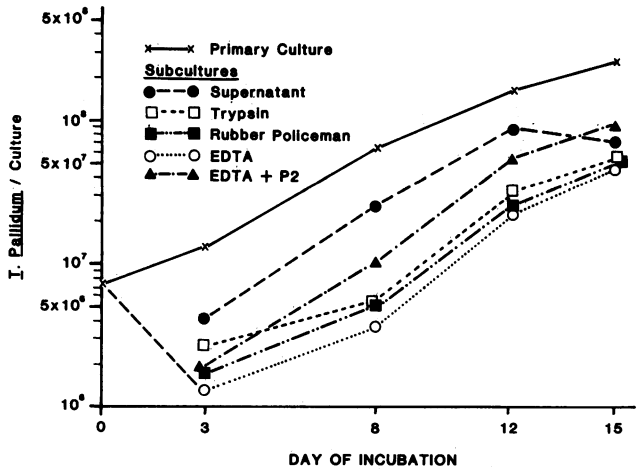

FIG. 1. Multiplication of *T. pallidum* in Sf1Ep rabbit epithelial cell cultures after subculture by five different procedures. Suspensions of treponemes from day 3 primary cultures were prepared as described in the text and transferred to secondary cultures. The number of *T. pallidum* in the primary and secondary cultures was determined 5, 9, and 12 days after subculture, corresponding to days 8, 12, and 15 of primary culture. Values represent the mean of six determinations on duplicate cultures. Standard deviations averaged 16% of the mean (range, 2 to 39%), with the highest values resulting from variability between duplicate subcultures.

TABLE 2. Multiplication of *T. pallidum* in secondary cultures originated by five subcultivation procedures

Expt	Day of subculture	Maximal fold increase (total fold increase)[a]						
		Primary culture		Secondary culture after subculture with:				
		Before subculture	After day of subculture	Mechanical disruption	Supernatant	Trypsin + EDTA	EDTA	EDTA + P2
1	3	1.8	21 (39)	32 (58)	22 (34)	21 (38)	37 (67)	55 (99)
2	3	1.4	21 (28)	16.7 (28)	32 (44)	—[b]	18 (24)	21 (29)
3	3	2.5	6.9 (17.5)	18.1 (46)	—	—	—	—
4	5	4.9	4.5 (22)	7.1 (35)	—	—	4.3 (21)	—
5	5	4.3	6.5 (19)	4.7 (20)	—	2.2 (9)	4.2 (18)	4.7 (20)
Mean ± SE		3.0 ± 0.8	12 ± 4 (25 ± 4)	16 ± 5 (36 ± 8)	27 (39)	12 (24)	16 ± 8 (33 ± 13)	27 ± 18 (49 ± 31)

[a] Total fold increase = fold increase in primary cultures before subculture times the maximal increase obtained in primary or secondary cultures after subculture.
[b] —, Not done.

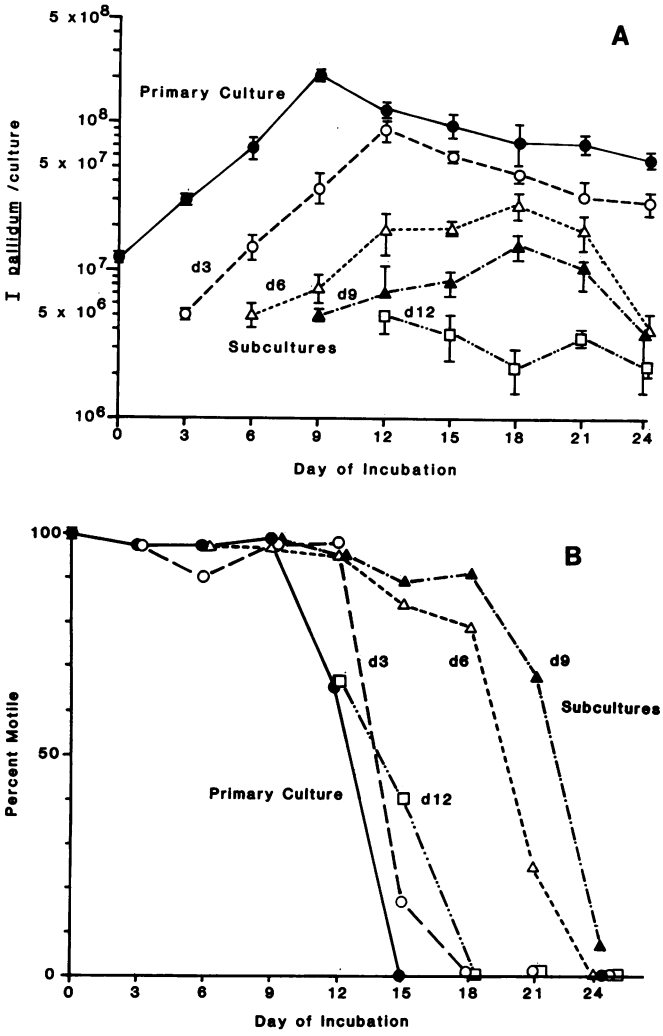

FIG. 2. Multiplication and motility of *T. pallidum* in primary cultures and in subcultures inoculated with treponemes from day 3, 6, 9, and 12 primary cultures. At the times indicated, the Sf1Ep cell monolayer in primary cultures was disrupted with a rubber policeman, and the resulting *T. pallidum* suspension was inoculated into fresh Sf1Ep cell cultures. Duplicate cultures for each time point and condition were then trypsinized and examined to determine number of *T. pallidum* and the percentage of motile organisms as a measure of viability. (A) Mean yield of *T. pallidum* per culture ± standard deviation. (B) Mean percentage of motile *T. pallidum*.

Time of subcultivation. The effect of time of subculture upon the multiplication of *T. pallidum* in secondary cultures was examined by transferring organisms by the mechanical disruption technique on days 3, 6, 9, and 12 of primary culture. A progressive decrease in the ability of *T. pallidum* to divide when placed in fresh secondary cultures was observed as the time of subculturing increased (Fig. 2A). However, the total fold increase obtained in subcultures established on days 3, 6, and 9 was somewhat greater than that of the primary cultures (Table 3). The maximal fold increase in the primary cultures was 17.5 (or 4.1 generations), compared with total increases of 45.3, 32.5, and 52.5-fold (5.5, 5.0, and 5.7 generations, respectively) in the organisms subcultured on days 3, 6, and 9, respectively.

Motility. In the subcultivation experiments, motility was utilized as a measure of viability. Treponemal motility was maintained for longer periods in day 3, 6, and 9 subcultures than in the primary cultures (Fig. 2B). For example, 63 and 68% of the cultured *T. pallidum* were still motile after 21 days of incubation in day 6 and 9 subcultures, respectively, whereas no motile organisms were observed in the primary cultures beyond day 12. Similar results were obtained in the experiments shown in Table 2, with primary cultures exhibiting a mean motility of 21 ± 10% (standard error of the mean) after 14 to 17 days of culture, compared with 67 ± 6% for the subcultures during the same time period. No consistent differences in the retention of motility were observed with the different subculture methods (data not shown).

Frozen *T. pallidum* as inoculum. In all previous experiments, the *T. pallidum* inoculated into Sf1Ep cultures had been freshly extracted from infected rabbit testes. For

TABLE 3. Effect of time of subculture on multiplication of *T. pallidum*

Culture	Maximal fold increase		
	Before subculture	Secondary culture	Total (primary × secondary)
Primary			17.5
Secondary initiated on day:			
3	2.5	18.1	45.3
6	5.7	5.7	32.5
9	17.5	3.0	52.5
12	17.5	0.75	13.1

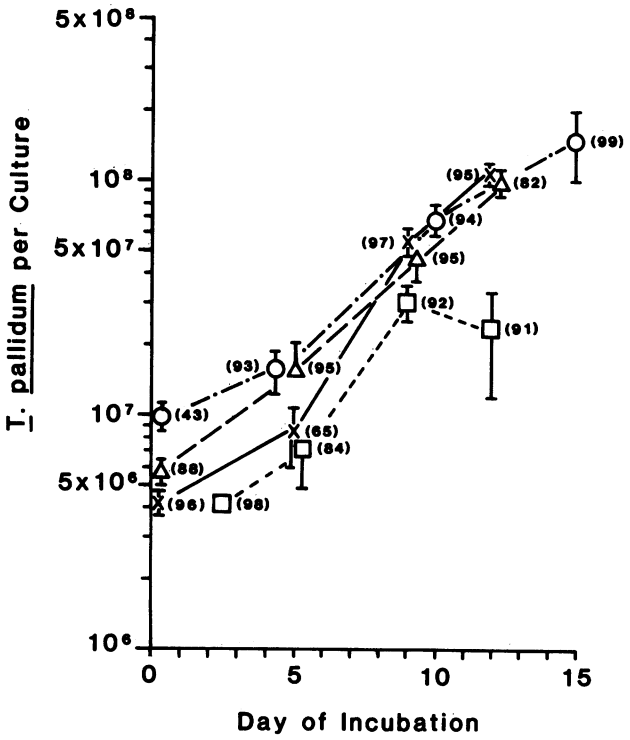

FIG. 3. Growth of *T. pallidum* in Sf1Ep cell cultures following storage at −70°C. In four separate experiments, *T. pallidum* were extracted from infected rabbit testes and then stored at −60°C for 25 to 91 days before thawing and inoculation into cultures. Excellent multiplication with good retention of motility was obtained in three of the four experiments. Each time point represents the mean yield per culture ± standard deviation of duplicate or triplicate cultures. The mean percentage of motile organisms is indicated in parentheses.

propagation of *T. pallidum* in rabbits, *T. pallidum* which has been stored at −70°C in phosphate-buffered saline with 50% normal rabbit serum, 1 mM DTT, and 15% glycerol is usually used as the inoculum. To determine whether treponemes stored in this manner could multiply in the in vitro culture system, frozen preparations of *T. pallidum* were thawed and added to Sf1Ep cell cultures prepared in the usual way and supplemented with filter-sterilized testes extract (0.34 ml), either freshly prepared or stored at −20°C. The frozen *T. pallidum* multiplied well in three of four experiments (Fig. 3), exhibiting increases of 25.9-, 17.3-, 15.4-, and 5.7-fold. Also, the percent motility was high (mean, 93%; range, 82 to 99%) on days 9 to 15, although motility at the time of inoculation or on day 5 was low (43 and 65%) in two experiments. The increase in percent motility in these experiments was presumably due to multiplication of viable treponemes, resulting in the dilution of organisms which were damaged during freezing and thawing.

DISCUSSION

Although continuous in vitro culture of *T. pallidum* has not yet been achieved, an understanding of the basic parameters affecting treponemal multiplication is gradually emerging. In this study, it was verified that culture conditions present in the *T. pallidum* cultivation system are not static but are constantly changing, and it is likely that many of these changes contribute to the cessation of treponemal growth. As the number of *T. pallidum* increases, several culture parameters, including Sf1Ep cell numbers, pH, redox potential, oxygen concentration, and glucose levels, are also undergoing alterations. It is likely that the changes in medium parameters are due to the metabolism of both the tissue culture cells and the bacteria, although the relative contributions of each have not yet been determined. Not only are the changes in the measured parameters likely to affect treponemal survival and growth, but they also indicate that other changes (such as depletion of amino acids, vitamins, and serum-derived nutrients and accumulation of toxic products) are occurring simultaneously. Thus, it is unlikely that adding or changing the concentration of individual medium components will significantly prolong multiplication in primary cultures, since depletion of other nutrients or creation of toxic conditions will eventually limit growth. An obvious solution to this problem is to stabilize culture conditions through continuous-flow medium replacement, and this approach is currently under investigation.

Another possible approach is the transfer of *T. pallidum* to fresh cultures. As shown in this study, *T. pallidum* subcultured by a number of different techniques can survive and proliferate in secondary cultures, demonstrating the feasibility of subcultivation. However, the ability of the subcultured treponemes to replicate in secondary cultures decreased with increasing age of the primary culture. Thus the in vitro-cultured organisms are apparently undergoing physiological changes which decrease their replicative potential, i.e., their ability to grow and divide when transferred to otherwise permissive growth conditions. Multiplication in subcultures originated on days 3 or 5 was roughly equivalent to (and in some cases exceeded) that in the primary cultures, indicating that irreversible damage had not occurred at that time. It could be argued that the total fold increase in the secondary cultures should be consistently and significantly greater than that of the primary cultures. However, the same changes in culture conditions shown in Table I also should occur in subcultures and thus limit growth, although medium conditions were not monitored in the subcultivation experiments.

Serial subculture at short (3 to 5 day) time intervals may permit prolonged treponemal survival and growth, and this technique will be tested in the near future. Its usefulness may be limited by the small number of divisions occurring between transfers, unless the subcultivation interval can be extended by improving culture conditions.

The ability of *T. pallidum* to thrive in infected tissue compared with the limited growth obtained in the current culture system may reflect important differences between the in vivo milieu and the tissue culture environment. One such difference is that conditions in vivo are relatively homeostatic, whereas in vitro culture conditions change rapidly, as discussed previously. Second, the extremely high cell density present in tissue may enhance treponemal growth by providing important nutrients or enzymatic activities. The loss of growth potential in the relatively low-density tissue culture system could be due to a lack of critical nutrients present in vivo but absent or rapidly lost during in vitro culture. This deficiency could result in slow "starvation" of the treponemes in culture, thus limiting the number of possible divisions. Third, a deficiency in protective mechanisms (including the quenching of oxygen radicals) which are provided by the surrounding tissue in vivo could also result in cumulative damage (such as DNA breakage [9]) in the organisms in culture due to oxygen toxicity or to other processes. Culture homeostasis through

continuous-flow medium replacement combined with high-density tissue culture techniques thus may provide a tissue like environment capable of maintaining continued growth of this fastidious organism.

In the final series of experiments reported, it was shown that *T. pallidum* stored in a frozen state was capable of multiplying in the Fieldsteel culture system. This finding enhances the feasibility of using primary cultures for large-scale production of *T. pallidum* for research or diagnostic purposes because it eliminates the need to continuously maintain *T. pallidum* infection in rabbits as a source of organisms for culture. Thus, the number of animals required would be reduced. However, the requirement for testicular tissue extract (3) is still a limiting factor, and identification of the growth-stimulating factor(s) it contains would further increase the potential of this system as a means of producing *T. pallidum* in large quantities.

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